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Research Paper

Anti-angiogenic properties of metronomic topotecan in ovarian carcinoma

William M. Merritt,[1,†] Christopher G. Danes,[1,†] Mian M.K. Shahzad,[1,6] Yvonne G. Lin,[1] Aparna A. Kamat,[1] Liz Y. Han,[2] Whitney A. Spannuth,[1] Alpa M. Nick,[1] Lingegowda S. Mangala,[1] Rebecca L. Stone,[1] Hye Sun Kim,[1] David M. Gershenson,[1] Robert B. Jaffe,[5] Robert L. Coleman,[1] Joya Chandra[4] and Anil K. Sood[1,3,*]

[1]Department of Gynecologic Oncology; [3]Department of Cancer Biology; and [4]Department of Pediatrics Research; U.T.M.D. Anderson Cancer Center; Houston, TX USA; [2]Departmen of Gynecolgic Oncology; U.T Southwestern Medical Center; Dallas, TX USA; [5]Center for Reproductive Sciences; University of California; San Francisco, CA USA; [6]Baylor College of Medicine; Department of Obstetrics and Gynecology; Houston, TX USA

†These authors contributed equally to this work.

Abbreviations: $CoCl_2$, cobalt chloride; cDNA, complimentary deoxyribonucleic acid; FBS, fetal bovine serum; Hif, hypoxia inducible factor; HUVEC, human umbilical vascular endothelial cell; MDACC, M.D. Anderson Cancer Center; MTD, maximum tolerated dose; MVD, microvessel density; PBS, phosphate buffered saline; RNA, ribonucleic acid; RT, room temperature; RT-PCR, reverse-transcriptase polymerase chain reaction; siRNA, small-interfering RNA; VEGF, vascular endothelial growth factor

Key words: ovarian, metronomic, orthotopic, topotecan, angiogenesis

Purpose: **Metronomic chemotherapy regimens have shown anti-tumor activity by anti-angiogenic mechanisms, however, the efficacy of metronomic topotecan in ovarian cancer is not known and the focus of the current study.**

Experimental design: **In vivo dose-finding and therapy experiments with oral metronomic topotecan were performed in an orthotopic model of advanced ovarian cancer. Tumor vascularity (MVD: CD31), proliferation (PCNA) and apoptosis (TUNEL) were examined among treatment arms. In vitro experiments including MTT and western blot analysis were performed to identify specific anti-angiogenic mechanisms of topotecan.**

Results: **Compared to controls, metronomic (0.5, 1.0 and 1.5 mg/kg; daily) and maximum tolerated therapy (MTD; 7.5 and 15 mg/kg; weekly) dosing regimens reduced tumor growth in dose-finding experiments, but significant morbidity and mortality was observed with higher doses. Metronomic and MTD topotecan therapy significantly reduced tumor growth in both HeyA8 and SKOV3ip1 models: 41–74% (metronomic), and 64–86% (MTD dosing) ($p < 0.05$ for both regiments compared to controls). Compared to controls, the greatest reduction in tumor MVD was noted with metronomic dosing (32–33%; $p < 0.01$). Tumor cell proliferation was reduced ($p < 0.001$ vs. controls) and apoptosis increased in all treatment arms ($p < 0.01$ vs. controls) for both dosing regimens. Endothelial cells demonstrated a significantly higher sensitivity to topotecan using metronomic dosing versus MTD in vitro. Pro-angiogenic regulators Hif-1α and VEGF levels were reduced in vitro (HeyA8 and SKOV3ip1) with topotecan independent of proteasome degradation and topoisomerase I.**

Conclusion: **Metronomic topotecan may be a novel therapeutic strategy for ovarian carcinoma with significant anti-tumor activity and target modulation of key pro-angiogenic mediators.**

*Correspondence to: Anil K. Sood; Professor; Departments of Gynecologic Oncology and Cancer Biology; Unit 1362; P.O. Box 301439; Houston, TX 77230-1439 USA; Tel.: 713.745.5266; Fax: 713.792.7586; Email: asood@mdanderson.org

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Introduction

Ovarian cancer was the most common cause of death among all gynecologic cancers in the United States in 2008.[1] Most ovarian cancer patients have favorable responses following standard treatment of surgical cytoreduction and chemotherapy; however, a majority will eventually recur and ultimately die from this disease. This occurs, in part, due to the tumor cells developing resistance to the most effective regimens including platinum and taxane-based agents. For these reasons, there is an urgent need to develop better treatment strategies.

Standard chemotherapeutic regimens are designed to deliver the highest or maximum tolerated dose (MTD), which can be safely administered and generally repeated.[2] Due to indiscriminant effects on normal tissues, rest periods of usually 3–4 w are scheduled between treatments for recovery and to minimize additive toxicity. However, recent studies indicate that tumor-associated endothelial cells continue to proliferate and promote cancer growth between treatments.[3,4] Moreover, it is not uncommon for dose-intensive strategies to be associated with unexpected delays ("treatment-holidays") requiring dose-reduction or concomitant marrow support. Similar effects are also seen with standard regimens that are administered chronically. The degree to which these factors contribute to

acquired chemoresistance is of great concern and have prompted many investigators to consider alternative chemotherapy dosing schedules. One such strategy, metronomic dosing, involves the frequent administration of chemotherapeutics at substantially lower doses. Ideally, the strategy would result in reduced normal tissue toxicity and minimize "off-treatment" exposure resulting in an improved therapeutic ratio.[4] In ovarian and breast cancer, clinical studies point to the potential value of such an approach. For example, weekly taxane chemotherapy, a form of semi-metronomic dosing, is highly effective and well-tolerated in patients with recurrent breast carcinoma, including those with taxane-resistant disease.[5-7] In this respect, the major therapeutic impact of metronomic dosing may lie in an alternative target, in the tumor associated endothelial cell. Contemporary investigative treatment programs have, in addition to targeting tumor cells, begun to focus on inhibiting tumor angiogenic pathways as a new method for decreasing cancer growth and spread. Since this concept was first presented by Folkman over 35 y ago,[8] numerous agents have been designed to specifically inhibit key angiogenic factors. Metronomic dosing of cytotoxic agents function as "anti-angiogenic" because the frequent, low dose administration appears to differentially target endothelial cells.[9,10]

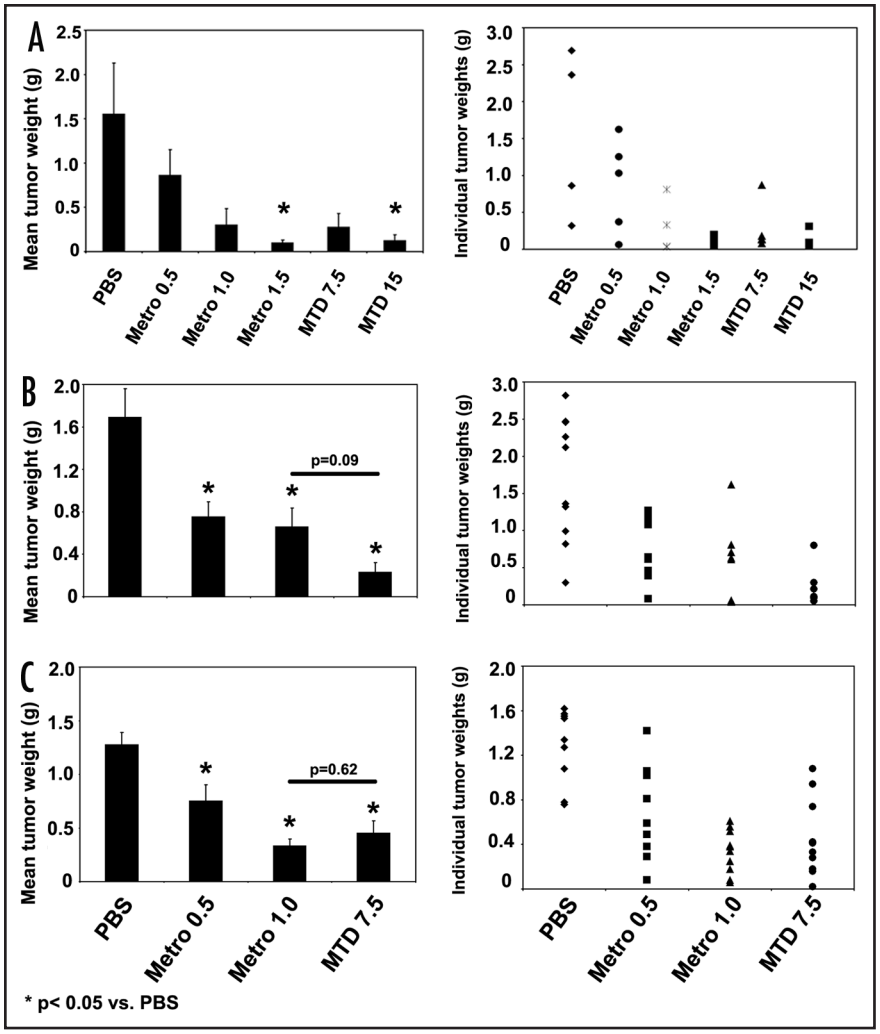

Figure 1. Effect of metronomic versus MTD topotecan therapy in an ovarian cancer model. (A) Dose-finding experiment. Effect of metronomic topotecan therapy on HeyA8 (B) and SKOV3ip1 (C) models. Mean tumor weights (left) and tumor weight distribution (right) are shown. Error bars represent s.e.m. *p < 0.05 compared to the PBS group.

Tumor angiogenesis is regulated by a balance of stimulatory and inhibitory factors modulated by both the tumor cells and the tumor microenvironment.[11] Among the stimulatory factors, hypoxia inducible factor (Hif) plays a critical role in hypoxia-mediated angiogenesis.[12] Hif-1 is comprised of two subunits, the constitutively expressed Hif-1β and the highly regulated Hif-1α. Deregulation of Hif-1α expression has been shown in several cancer types including ovarian cancer.[13-15] Because Hif-1α is so critical to several tumorigenic properties, it is an ideal target for cancer therapies. Topotecan, a semi-synthetic analogue of camptothecin, is a potent topoisomerase-I inhibitor[16] and is currently FDA approved in the US for the treatment of recurrent ovarian cancer and second-line small-cell lung cancer. However, in light of this mechanism, the agent is used in treatments for various other tumor types as well. Along with its cytotoxic effects, topotecan has been suggested to possess potent anti-angiogenic properties and is a Hif-1 antagonist.[17] Therefore, we chose to test the functional and biological effects of metronomic topotecan therapy in ovarian cancer models.

Results

Metronomic topotecan dose-finding in vivo. Prior to initiating in vivo therapy experiments with topotecan, we performed dose-finding trials in orthotopic murine models of ovarian cancer. One week after inoculation of female nude mice with HeyA8 ovarian cancer cells, we randomized treatment animals to one of the following groups: control (PBS), metronomic 0.5–1.5 mg/kg, and MTD 7.5–15 mg/kg dosing (five mice/arm). While all therapeutic regimens were effective in reducing tumor growth (Fig. 1A), metronomic 1.5 mg/kg (95% reduction vs. controls; p = 0.014) and MTD 15 mg/kg (89% reduction; p = 0.03) dosing resulted in greatest reduction of tumor growth. Compared to controls, metronomic 0.5 (50%; p = 0.46) and 1 mg/kg (68%; p = 0.08) and MTD 7.5 mg/kg dosing (74%; p = 0.05) also reduced tumor growth. The difference between the 1.0 mg/kg and the 1.5 mg/kg dosing groups was not statistically significant. During these experiments, tolerance to therapy was monitored. It was noted that mice treated with the higher doses (metronomic 1.5 mg/kg and MTD 15 mg/kg) were observed to be less active with decreased food and water intake.

Table 1 **Characteristics of tumors following topotecan therapy**

Cell line	Group	Tumor incidence (%)	Mean nodule count (95% CI)	p value (vs. controls)
HeyA8	PBS	100	7.6 (4.9–10.3)	
	Metronomic 0.5 mg/kg	90	4.2 (-1.5–10.0)	0.007
	Metronomic 1.0 mg/kg	80	2.5 (0.4–4.6)	0.002
	MTD 7.5 mg/kg	80	3.6 (0.7–6.5)	0.007
SKOV3ip1	PBS	90	30.8 (26.3–35.3)	
	Metronomic 0.5 mg/kg	100	16.7 (11.5–21.9)	0.05
	Metronomic 1.0 mg/kg	100	11.3 (7.7–14.9)	0.003
	MTD 7.5 mg/kg	100	10.3 (7.8–12.8)	0.006

For these reasons, we chose to use metronomic doses of 0.5 and 1.0 mg/kg versus MTD 7.5 mg/kg for all further therapy experiments.

Metronomic topotecan therapy in an orthotopic ovarian cancer model. To test the effects of metronomic topotecan therapy in ovarian cancer, we designed models utilizing two different ovarian cancer cell lines, HeyA8 and SKOV3ip1. Study arms (ten mice/arm) included control (PBS; 100 μL p.o. daily), metronomic topotecan 0.5 and 1 mg/kg (100 μL p.o. daily), and MTD 7.5 mg/kg (100 μL p.o. weekly). In both the HeyA8 and the SKOV3ip1 models, the lower metronomic dosing (0.5 mg/kg) reduced tumor weights by 41–55% as compared to PBS treated mice ($p = 0.02$ for both; Fig. 1B and C). The 1.0 mg/kg metronomic dose and the MTD 7.5 mg/kg doses also reduced tumor growth (61–74% and 64–86%, respectively, $p < 0.01$) when compared with mice treated with PBS. Among the treatment groups, there was no difference in tumor incidence (Table 1). There was also no statistical difference between MTD and metronomic dosing schedule in terms of tumor growth inhibition ($p > 0.05$).

Neither metronomic dosing regimen (0.5 or 1.0 mg/kg) demonstrated toxicity as determined by mouse weight at the completion of therapy. However, the mice in the HeyA8 model for the MTD (7.5 mg/kg) dose did have a significant decrease in mean mouse weight ($p < 0.01$ as compared to PBS treated controls). Observations by investigators and animal care staff did not note any differences in eating or drinking habits in the four treatment groups in both models.

Effects of metronomic topotecan on the tumor microenvironment. We next considered potential effects on the tumor microenvironment. Tumors from the therapy experiments above were examined for apoptosis, proliferation and microvessel density (MVD). Tumor cell apoptosis was compared among treatment groups by quantitative analysis of TUNEL staining of tumors from only the HeyA8 therapy group (Fig. 2A). Compared to tumors from control mice, metronomic therapy increased apoptosis two-fold (0.5 and 1.0 mg/kg; $p < 0.001$) while MTD increased apoptosis 2.5 fold ($p < 0.001$).

Tumor cell proliferation was examined in tumors from the HeyA8 and SKOV3ip1 therapy experiments by comparing the percentage of proliferating cells (PCNA) with respect to topotecan therapy. HeyA8 and SKOV3ip1 tumors from mice treated with 0.5 mg/kg metronomic therapy displayed a significant decrease in tumor cell proliferation as compared to controls (19–20%, $p < 0.001$; Fig. 2B). MTD had the greatest decrease in tumor cell proliferation as compared to controls in the HeyA8 model (30%, $p < 0.001$); however, was not significantly greater than the metronomic 1.0 mg/kg dosing (27% reduction vs. controls, $p < 0.001$). In the SKOV3ip1 model, 1.0 mg/kg metronomic therapy had the greatest effect on tumor cell proliferation (26% reduction vs. controls, $p < 0.001$), although was no different than MTD dosing relative to controls (22% reduction).

MVD was assessed using IHC staining for CD31 in both models. Compared to controls, all topotecan treatment arms significantly decreased MVD (Fig. 2C). Metronomic dosing (0.5 mg/kg) decreased MVD by 17–27% in both HeyA8 and SKOV3ip1 tumor models ($p < 0.05$). The higher metronomic dose (1.0 mg/kg) decreased MVD by 32–33% (HeyA8, $p < 0.001$ and SKOV3ip1, $p = 0.007$), while the MTD demonstrated a 28–30% reduction in MVD (HeyA8, $p < 0.001$ and SKOV3ip1, $p = 0.02$). Therefore, the greatest reduction in MVD was seen in the metronomic 1.0 mg/kg regimen though not statistically greater than MTD.

Effect of metronomic topotecan on tumor and endothelial cell viability in vitro. To test whether there was differential sensitivity of tumor (HeyA8) or human endothelial cells (HUVEC) to topotecan at MTD versus metronomic dosing, we analyzed cell viability of both cell lines following either single versus daily drug applications. The IC_{50} levels for each cell line and each treatment are represented in Table 2. There was no substantial difference between the IC_{50} for MTD and metronomic dosing in the HeyA8 cells (~25 nM for both). However, the sensitivity of HUVEC cells to topotecan was highly increased following metronomic dosing (1 vs. 12 nM).

Topotecan therapy modulates pro-angiogenic factors in ovarian carcinoma. We have shown that topotecan therapy was able to decrease MVD in vivo. It has been shown that in certain cancer models topotecan targets angiogenesis by downregulating pro-angiogenic factors Hif-1α and VEGF.[24-26] In vitro, topotecan reduced Hif-1α and VEGF protein levels in a dose-dependent manner in the HeyA8 and SKOV3ip1 cells (Fig. 3A). We also treated the HeyA8 and SKOV3ip1 cells with increasing doses of topotecan under hypoxic conditions (1% O_2), which showed similar results (data not shown). Hif-1α expression was examined by immunoprecipitation from protein extracted from tumors at the completion of the HeyA8 therapy experiment (Fig. 3B). Quantification of Hif-1α expression was compared by densitometry analysis of western blot imaging. Metronomic 1.0 mg/kg and MTD 7.5 mg/kg dosing demonstrated the greatest reduction,

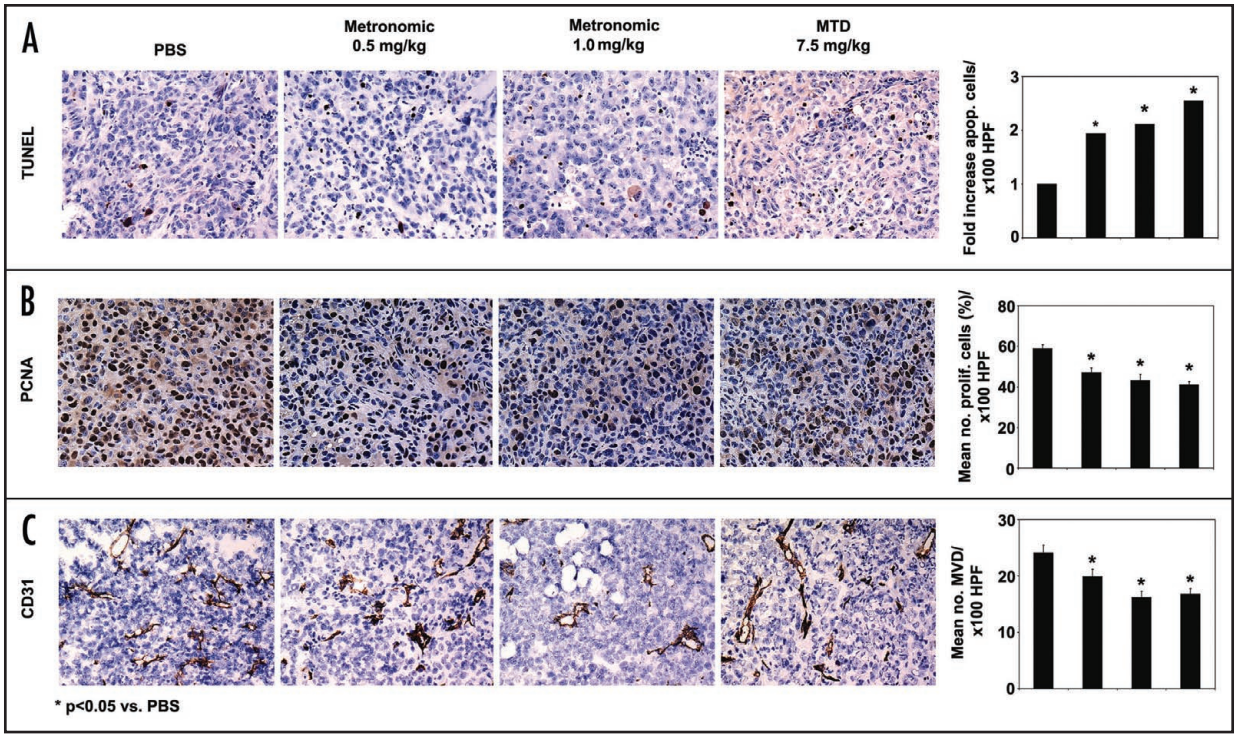

Figure 2. Immunohistochemical analyses of (A) apoptosis (TUNEL), (B) tumor cell proliferation (PCNA), and (C) microvessel density (CD31) following metronomic or MTD topotecan therapy in the HeyA8 model. Photographs were taken at original magnification x100. The bars in the graphs correspond to the labeled columns on the left. Error bars represent s.e.m. *p < 0.05 compared to the PBS group.

66% and 64%, respectively, in Hif-1α when compared to tumors from the mice treated with PBS. Immunohistochemical analysis of tumors from the HeyA8 model demonstrated a noticeable decrease in VEGF expression in both metronomic and MTD dosing therapy arms when compared to control tumors (Fig. 3C).

Metronomic topotecan regulates Hif-1α via non-classical mechanisms. First we sought to determine if topotecan mediated the transcription of Hif-1α. Using RT-PCR, we found no difference in the RNA levels of Hif-1α following treatment with topotecan under hypoxic conditions (Fig. 4A). Topotecan was developed as a semi-synthetic derivative of camptothecin, to specifically inhibit topoisomerase I (Topo1). Therefore, we tested whether topotecan mediated downregulation of Hif-1α was dependent on Topo1. HeyA8 and SKOV3ip1 cells were treated with Topo1 siRNA with or without topotecan. Hif-1α downregulation by topotecan (Fig. 4B) was independent of Topo1 silencing, suggesting that topotecan is working though a mechanism independent of Topo1.

Hypoxia mediated Hif-1α protein expression has been extensively studied as a pro-angiogenic mediator. Hif-1α protein levels are highly regulated via the proteasome degradation pathway. Under hypoxic conditions, Hif-1α ubiquitination via the von Hippel-Lindau protein is blocked and Hif-1α is stabilized, translocates to the nucleus and transcriptionally activates a critical set of proteins.[27] Therefore, we asked whether topotecan-mediated Hif-1α regulation requires proteasome mediated degradation. Treatment of cells with a proteasome inhibitor, MG132, revealed that downregulation of Hif-1α was not via proteasomal degradation (Fig. 4C).

Table 2 **Effect of metronomic and MTD topotecan therapy on the viability of ovarian cancer and human endothelial cells in vitro**

Cell lines	Topotecan IC_{50} (nmol/ L)	
	MTD	Metronomic
Ovarian cancer cell line		
HeyA8	25	24
Human endothelial cell line		
HUVEC	12	1

HUVEC, human umbilical vascular endothelial cell.

Discussion

The key findings of this study are that metronomic topotecan therapy was equally effective in reducing tumor growth in a murine model of advanced ovarian cancer when compared to conventional MTD dosing. The metronomic regimen was able to significantly decrease cell proliferation and angiogenesis while increasing tumor cell apoptosis. These anti-tumor effects were likely due to a reduction in the pro-angiogenic factors, VEGF and Hif-1α, which contributed to decreased angiogenesis following topotecan therapy.

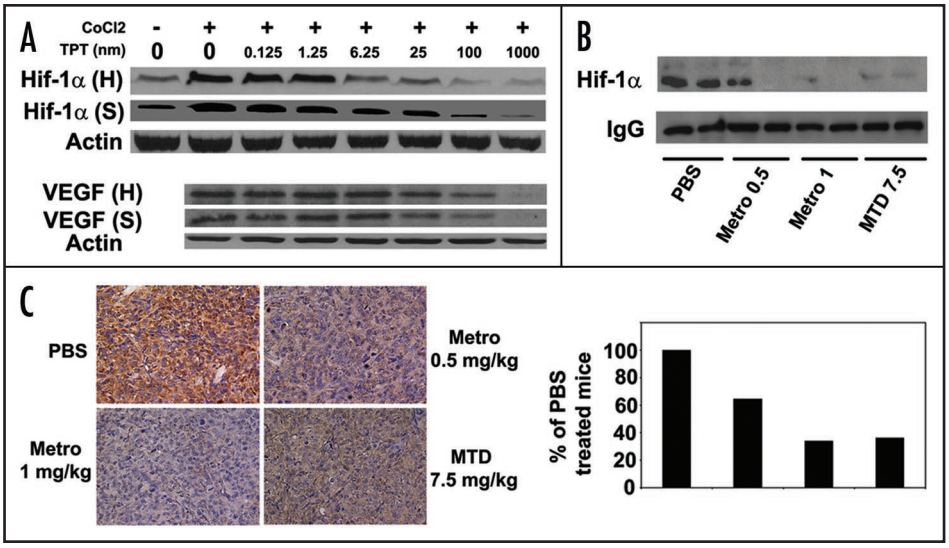

Figure 3. (A) Effect of topotecan on Hif-1α and VEGF with or without cobalt chloride ($CoCl_2$: 250 μm) on HeyA8 (H) and SKOV3ip1 (S) cells. (B) Immunoprecipitation and immunoblot for Hif-1α following topotecan therapy in HeyA8 tumors (densitometry shown below in graph). (C) Immunohistochemical staining for VEGF in HeyA8 tumors following metronomic or MTD topotecan (final magnification x100).

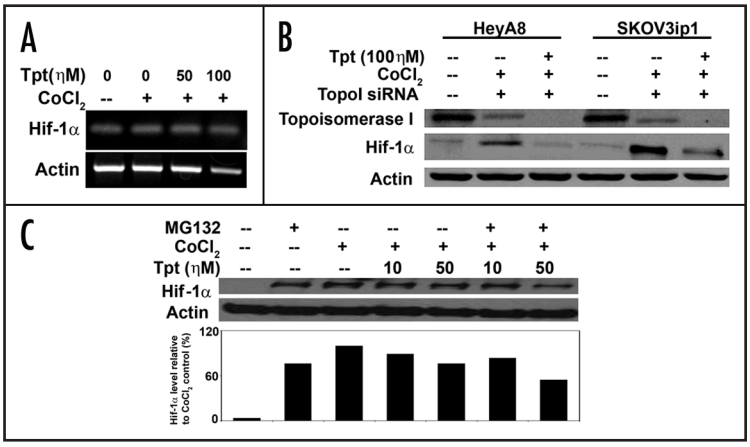

Figure 4. In vitro effects on Hif-1α in HeyA8 cells with and without $CoCl_2$ in presence of (A) topotecan (RT-PCR), (B) topoisomerase1 silencing and topotecan, and (C) SKOV3ip1 cells in presence of proteasomal inhibition (10 μM MG132 for 4 h) and topotecan.

Metronomic chemotherapy was first presented as an alternative to traditional chemotherapeutic dosing to overcome barriers of toxicity and resistance seen with traditional high-dose therapies. Early preclinical studies have shown metronomic dosing of agents, such as cyclophosphamide and vinblastine, to be as effective in tumor growth inhibition compared to MTD regimens.[28-30] Moreover, minimal toxicity was observed in mice treated with metronomic dosing.[29-31] Clinical trials have also demonstrated that frequent lower dosing has low toxicity.[32] Green and colleagues reported that dose-dense scheduling of weekly paclitaxel in combination with 5-FU, cyclophosphamide, and doxorubicin improved pathologic response rates in breast cancer patients compared to the same three agents given with paclitaxel therapy every 3 w.[6] Research on metronomic dosing regimens in ovarian cancer has been relatively limited, but we have previously demonstrated that metronomic taxane therapy was superior to MTD in both taxane-sensitive and -resistant ovarian cancer models.[19] Clinically, Garcia and colleagues recently reported that daily oral cyclophosphamide with bevacizumab led to a 24% overall response rate and survival of 17 mo in patients with recurrent ovarian carcinoma.[33] Our results show that metronomic topotecan therapy was as effective as MTD in reducing tumor growth with tolerable toxicity. Together, these data support the need to further investigate the therapeutic benefit of metronomic therapy with commonly used cytotoxic agents in cancer therapy.

The rationale behind continual low-dose therapy was based on the theory that decreased rest between treatments would enhance endothelial cell death while maintaining equal tumor cell kill.[29,30] Studies have shown that endothelial cells were much more sensitive to chemotherapeutic agents in vitro and vivo when given metronomic versus traditional dosing.[31] Metronomic dosing in ovarian cancer models significantly reduced tumor vascularity and tumor cell proliferation compared to tumors from control mice.[19] Here, we show evidence that would support this research in that metronomic dosing with topotecan had similar effects on tumor cell survival through significantly enhanced endothelial cell sensitivity to topotecan therapy in vitro.

Topotecan therapy has been shown to be an effective agent in ovarian cancer patients when given in weekly regimens;[34] however, rest periods are often needed to prevent cumulative toxicities. For example, Rose and colleagues investigated the benefit of alternating oral topotecan and etoposide, a topoisomerase II inhibitor. Patients were treated daily for approximately one week with each agent on 28 d cycles. Although only modest response rates were observed, the regimen was well tolerated with minimal toxicity.[35] Topotecan has also shown anti-angiogenic effects in both in vitro and in vivo studies.[17,36] Using a disc angiogenesis system (DAS), Clements and colleagues reported that intramuscular injection of topotecan (1 mg/kg) given every other day for 14 d reduced vascularity by 30%.[17] Moreover, topotecan is very unstable under physiological conditions, having a half life in the active form of only 2.8 h.[37] In vitro experiments longer than 24 h required that the topotecan be replaced every 24 h to sustain the molecular effect (data not shown). The transience of active topotecan makes it an ideal therapeutic for a metronomic regimen. Here, we demonstrated that metronomic topotecan was highly effective in reducing human endothelial cell viability in vitro and tumor vascularity in a murine ovarian cancer model. Our data do

not support the hypothesis that metronomic dosing of topotecan is more effective than MTD therapy in vivo; however, given the similar efficacy in reducing tumor vascularity and tumor cell proliferation, we feel that metronomic therapy provides a novel regimen with tolerable toxicities to further explore in ovarian cancer.

Tumor angiogenesis is a result of the interaction of several pro- and anti-angiogenic factors. In addition to the direct effects of metronomic dosing on tumor-associated endothelial cells, certain chemotherapeutic agents have also been shown to modulate key mediators of the angiogenesis pathway, which would support the anti-angiogenic benefit of metronomic scheduling over traditional dosing. For example, metronomic dosing of cyclophosphamide significantly increased plasma levels of thrombospondin-1 (TSP-1), an anti-angiogenic factor, in murine cancer models when compared with MTD dosing.[38] Topotecan has been shown to affect the expression of pro-angiogenic factors.[39,40] A screen of approximately 2000 compounds that was designed to find modulators of the Hif-1α protein found that topotecan was a potent inhibitor of Hif-1α transcriptional activation.[24] In neuroblastoma cells, topotecan was able to decrease the transactivation and secretion of VEGF by inhibiting the accumulation of Hif-1α and Hif-2α.[40] There is currently a pilot trial ongoing for daily oral topotecan to treat refractory advanced solid tumors expressing Hif-1α (Protocol # 05-C0186). In the current study, we found that metronomic topotecan was able to decrease the expression of Hif-1α in vitro and in vivo. We show that the mechanism by which this occurs is independent of transcriptional regulation. Furthermore, we demonstrated that topotecan decreased Hif-1α levels in a dose-dependent fashion, independent of the classical proteasome degradation pathway and of Topo1. The underlying mechanism behind this pathway remains unknown; however, we are currently studying the regulation of Hif-1α at the translational level.

Another potential benefit of metronomic chemotherapy is the opportunity to design combination regimens with other therapeutic agents. Preclinical models demonstrated that metronomic dosing in combination therapy may have superior efficacy in chemoresistant models.[19,28] Colleoni and colleagues demonstrated that low-dose methotrexate in combination with cyclophosphamide had significant anti-tumor effects with low toxicity in patients with metastatic breast cancer.[41] Several combination therapies have been developed using metronomic therapy in conjunction with novel anti-angiogenic agents.[19,28,33,42] Due to the high rate of tumor recurrence and chemoresistance in ovarian cancer patients, metronomic therapy may be a reasonable alternative to conventional regimens. Here, we provide evidence that metronomic and MTD therapies lead to significant tumor growth inhibition with moderate anti-angiogenic effects. Together, these data suggest that metronomic topotecan may be even more efficacious than MTD when combined with other agents due to lower toxicity, a common treatment delaying factor. Currently, we are exploring the benefit of combining metronomic topotecan therapy with novel small molecular inhibitors of VEGF receptor kinase activity.

In summary, we have demonstrated that metronomic topotecan therapy is highly effective in reducing tumor growth in a murine model of advanced ovarian cancer. This regimen, although similar to MTD dosing, reduced tumor vascularity and cellular proliferation with minimal observed toxicity. Anti-angiogenic properties of metronomic topotecan were also demonstrated by the enhanced sensitivity of topotecan therapy on human endothelial cells in vitro. Furthermore, topotecan significantly decreased the expression of potent pro-angiogenic cytokines, VEGF and Hif-1α, in vitro and vivo, which would suggest an indirect effect on the tumor microenvironment.

Methods

Cell lines and cultures. The derivation and source of the human epithelial ovarian cancer cell lines HeyA8 and SKOV3ip1 have been described previously.[18] Cell lines were maintained and propagated in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). Human umbilical vascular endothelial cells (HUVECs) were maintained in DMEM with 10% FBS and 10% basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO). All experiments were performed using cells grown to 60–80% confluence, and all cell lines were routinely tested to confirm absence of Mycoplasma.

Cell viability assays. To compare the sensitivities between MTD and metronomic dosing of topotecan (GlaxoSmithKline, Philadelphia, PA) on ovarian cancer cells and human endothelial cells, 2,000 cells were plated per well into a 96-well plate and allowed to adhere overnight. Each experimental condition was performed in triplicate to confirm findings. Cells were treated with either a single dose (MTD) or daily (metronomic) topotecan therapy (HeyA8: 0.1 nM–1 μM; HUVEC: 0.01 nM–500 nM). On day 5 (HeyA8) and day 9 (HUVEC), cell viability was assessed by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich), as previously described.[19]

Orthotopic model of ovarian cancer. Female athymic nude mice (NCr-*nu*) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. The mice were cared for in accordance with guidelines set forth by the American Association for Accreditation for Laboratory Animal Care and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the MDACC Institutional Animal Care and Use Committee.

The development and characterization of the orthotopic model of advanced ovarian cancer used in these experiments has been previously described by our laboratory.[20,21] Briefly, human ovarian cancer cells were grown and prepared, as previously described.[22] Each mouse was injected intraperitoneally with 200 μL of cell suspension. An in vivo topotecan dose-finding experiment was performed by treating (five mice/group) with oral suspensions of PBS (control), metronomic topotecan (daily) doses of 0.5, 1.0, 1.5 mg/kg or weekly (maximum tolerated dose: MTD) doses of 7.5 and 15 mg/kg. Tumor weights were recorded for all mice during necropsy. Therapy experiments were performed in HeyA8 and SKOV3ip1 models comparing control (PBS), metronomic, and

MTD therapy regimens (ten mice/group). The timing of necropsy was determined when any mouse from either the control or a treated group became moribund (which ever occurred first) and was approximately 28 d after HeyA8 and 38 d after SKOV3ip1 cell injection. Tumors were harvested and fixed in formalin for paraffin embedding or snap-frozen in optimal cutting medium (OCT; Miles, Inc., Elkhart, IN) for immunohistochemical analyses.

Immunohistochemistry. Immunohistochemical analyses of CD31, proliferating cell nuclear antigen (PCNA), terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), vascular endothelial growth factor (VEGF) were conducted on 4 μm-thick frozen or formalin-fixed paraffin-embedded orthotopic ovarian cancer specimens as described previously.[19,23] Primary antibodies used included: VEGF (rabbit polyclonal anti-human, 1:25 dilution; Biosource, Camarillo, CA); PCNA (PC-10 mouse monoclonal IgG, 1:50 dilution; Dako, Carpinteria, CA); or CD31 (rat monoclonal anti-mouse, 1:800 dilution; BD Bioscience, Pharmingen, San Jose, CA). Quantification of microvessel density (CD31), tumor cell proliferation (PCNA) and apoptosis (TUNEL) was performed on slides from each treatment group, as previously described.[19,22]

Immunoprecipitation/western blot assays. Cell lysates were obtained with RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton, 0.5% deoxycholate, 25 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mM EDTA and 1 mM sodium orthovanadate), centrifuged at 13,000 rpm for 10 min at 4°C and protein concentrations were determined with the use of a Protein Assay kit (Bio-Rad, Hercules, CA). Following protein loading, bands were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose paper, and blocked with 5% milk for 2 h at room temperature (RT). Blots were incubated overnight at 4°C with either VEGF (1:1,000 dilution; R&D Abs, North Las Vegas, NV) or Hif-1α antibody (1:750 dilution; BD Biosciences, San Jose, CA), Topoisomerase-1 (1:1,000, SCBT, Santa Cruz, CA), Actin (1:10,000, Sigma, St. Louis, MO) washed with PBS, and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:2,000; GE Healthcare UK Limited, Buckinghamshire, UK) for 2 h at RT. Blots were developed with the ECL western blotting Detection Kit (GE Healthcare).

To measure Hif-1α expression following topotecan therapy in vivo, a portion of tumors from the HeyA8 therapy experiment was incubated on ice in RIPA lysis buffer for 20 min, homogenized, and centrifuged at 13,000 rpm for 12 min at 4°C and then protein concentrations of the resulting supernatants were determined as described above. Immunoprecipitation of 1,000 μg of protein in cell lysate was performed by incubation with a Hif-1α antibody for 12 h at 4°C. Protein A sepharose beads (60 μL of a 1:1 dilution in PBS; Upstate Cell Signaling, Lake Placid, NY) were then added and the mixture was incubated for 4 hours at 4°C. Samples were then centrifuged at 1,000 rpm at 4°C for 1 min, washed with PBS, and resuspended in equal amount of PBS/Laemmli buffer. Western blot assays for Hif-1α expression were then performed as described above. Immunoblots were quantified by densitometry analysis using Scion Image 0.4.0.3 (Scion Corporation, Frederick, MD).

Reverse transcript polymerase chain reaction (RT-PCR). Ovarian cancer cell line HeyA8 was grown on 10 cm plates and treated with/without $CoCl_2$ (250 μM) and increasing concentration of topotecan (50 and 100 nM) for 48 h. RNA was isolated from the cells using RNeasy Mini-kit (Qiagen, Valencia, CA). Following quantitation, 4 μg of RNA was used to make cDNA with the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers specific for Hif-1α and Actin were used for PCR (sequences available upon request). cDNA was then separate using a 1.5% agarose gel and stained using ethidium bromide. The gel was visualized using a Foto/Analyst PC Imaging System (Fotodyne Inc., Hartland, WI).

Hypoxia treated cells/proteasome inhibition. HeyA8 and SKOV3ip1 cells were grown on 10 cm plates and treated with increasing doses of topotecan (0.125, 1.25, 6.25, 25, 100, 1,000 nM) with hypoxia mimetic $CoCl_2$ (250 μM) for 24 h. Lysates were collected for analysis by western blot. For the proteasome inhibitor experiment, SKOV3ip1 cells were treated as above with 10 and 50 nM topotecan and $CoCl_2$ for 24 h. Four hours prior to collection, the cells were treated with 10 μM of the proteasome inhibitor MG132 (Sigma, St. Louis, MO). The cells were then collected, lysed and analyzed by western blot.

SiRNA constructs and in vitro delivery. SiRNA (Qiagen, Valencia, CA) was predesigned to silence topoisomerase-I (Topo1) expression (Cat #SI02662933). For in vitro delivery, siRNA (10 μg) was incubated with 30 μL RNAiFect transfection reagent (Qiagen) for 10 min at RT and added to HeyA8 or SKOV3ip1 cells in culture at 80% confluence in 10 cm culture plates. The cells were treated with topotecan (100 ηM) and $CoCl_2$ (250 μM) for 48 h as above. The cells were then collected, lysed and analyzed by western blot.

Statistical analysis. For animal experiments, ten mice were assigned per treatment group. This sample size gave 80% power to detect a 50% reduction in tumor weight at a 5% level of statistical significance. Mouse and tumor weights and the number of tumor nodules for each group were compared using Student's t-test (for comparisons of two groups) and analysis of variance (for multiple group comparisons). Normality of weight distributions was tested by Kolmogrov-Smirnov test. All statistical tests were two-sided and p values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 12 for Windows statistical software (SPSS, Inc., Chicago, IL).

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